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Supplemental Information

C. elegans S6K Mutants Require a Creatine-Kinase-like Effector for Lifespan Extension

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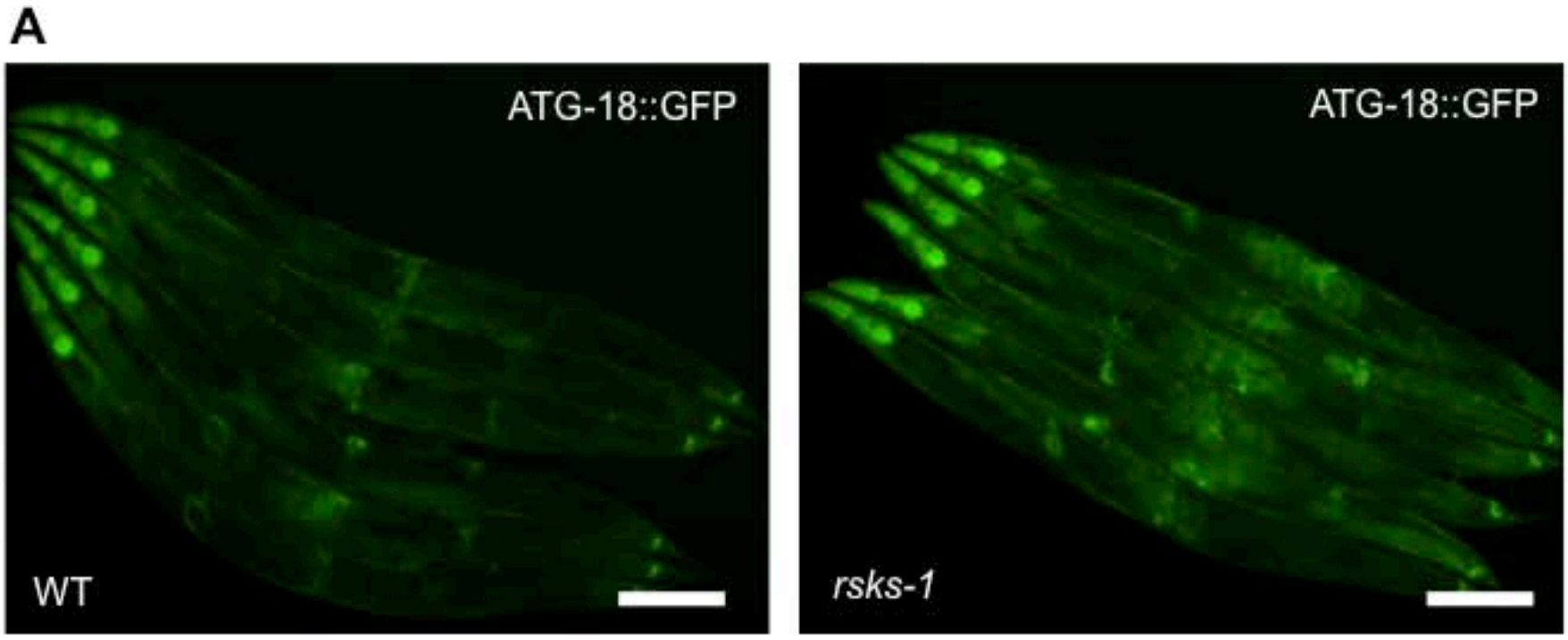

Figure S1. ATG-18 is Increased in *C. elegans rsks-1*/S6K Mutants; Related to Experimental Procedures.

(**A**) Fluorescence micrographs of 2-day old wild-type (N2, WT) or *rsks-1(sv31)* animals stably expressing GFP-tagged ATG-18 from the *atg-18* promoter. Scale bars, 300 µm.

(**B**) Quantitative RT-PCR of total RNA from 1-day-old WT and *rsks-1(sv31)* animals. mRNA levels of *gst-4, atg-18,* and *argk-1* were normalized to housekeeping genes *ama-1, cyn-1, nhr-23* and *pmp-3* and are depicted relative to WT. Mean + SEM of three biological samples. The different mRNA levels in *rsks-1(sv31)* were compared to WT using Student's *t*-test: $^{**}p < 0.01$.

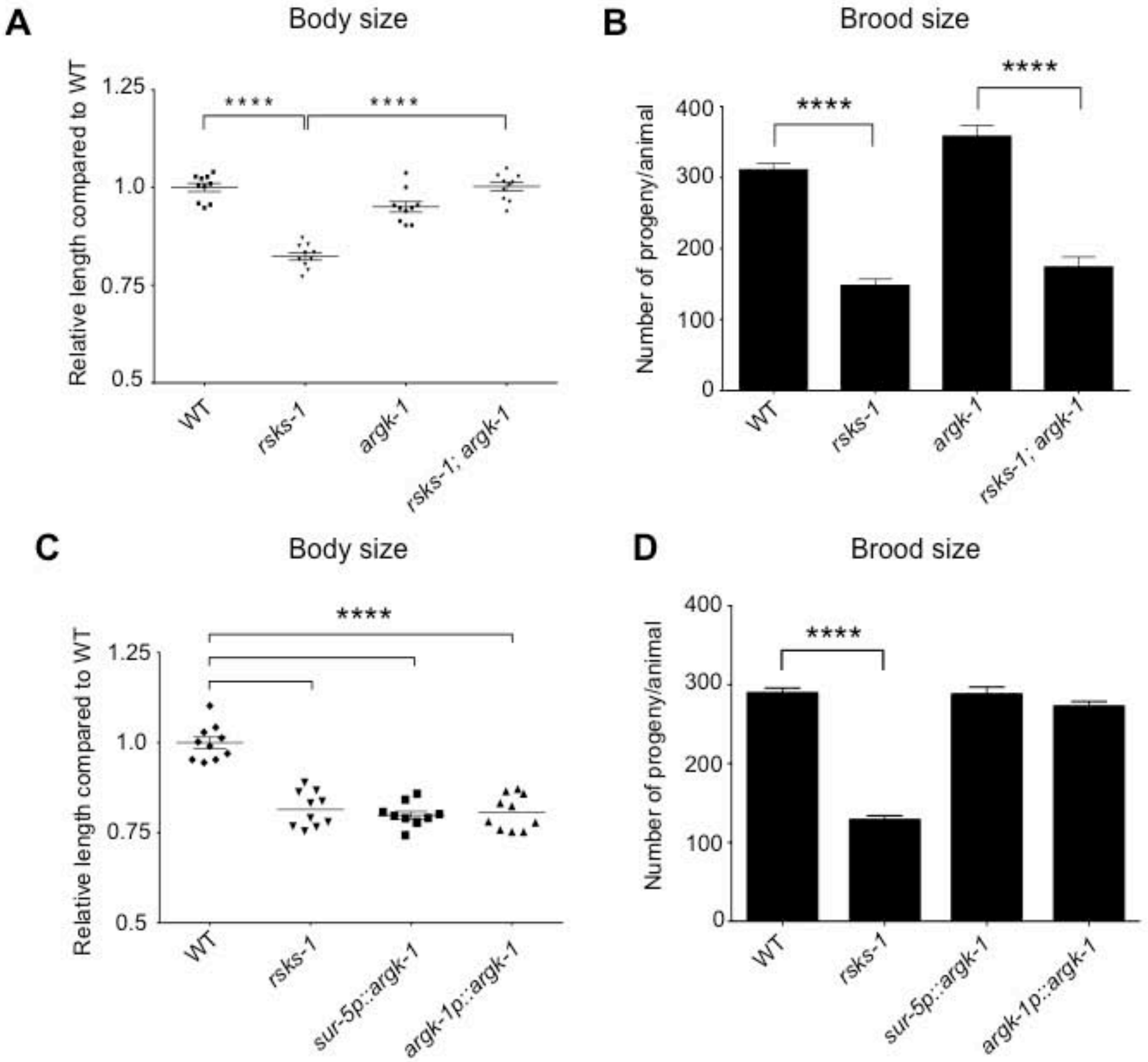

Figure S2. Body and Brood Size Phenotypes in *rsks-1*/S6K Mutants With and Without *argk-1*; Related to Experimental Procedures.

(**A**) Average body length of day-1 adult wild-type (WT, N2), *rsks-1(sv31)*, *argk-1(ok2993)*, and *rsks-1(sv31); argk-1(ok2993)* animals. N = 10 animals; error bars are SEM. ****p < 0.0001 by one-way ANOVA. Scale bars, 200 μm. Animals were raised at 20°C, and the experiment was repeated once with similar results.

(**B**) Average number of progeny per animal was determined for the same strains as in (**A**). N = 10 animals; error bars are SEM. ****$P < 0.0001$ by one-way ANOVA. The experiment was repeated once with similar results. In both assays, the results obtained with *rsks-1(sv31)* and *rsks-1(sv31); argk-1(ok2993)* were not significantly different.

Figure S2 (continued):

(**C**) Average body length of WT, *rsks-1(sv31)*, or transgenic animals expressing ARGK-1 from the ubiquitous *sur-5* promoter (*sur-5p::argk-1::gfp*, Line A) or from the endogenous *argk-1* promoter (*argk-1p::argk-1::mcherry*, Line A) measured on Day 1 of adulthood. N = 10 animals; error bars are SEM. ****p < 0.0001 by one-way ANOVA. Animals were raised at 20°C. The experiment was repeated once with similar results.

(**D**) Average number of progeny per animal was determined for the same strains as in (**C**). N = 10 animals; error bars are SEM. ****p < 0.0001 by one-way ANOVA. The experiment was repeated twice with similar results. While repeating these experiments, we observed that *sur-5p::argk-1::gfp* animals (Line A) gave lower brood size at least once after cultivating these animals for a period of time following thawing (data not shown).

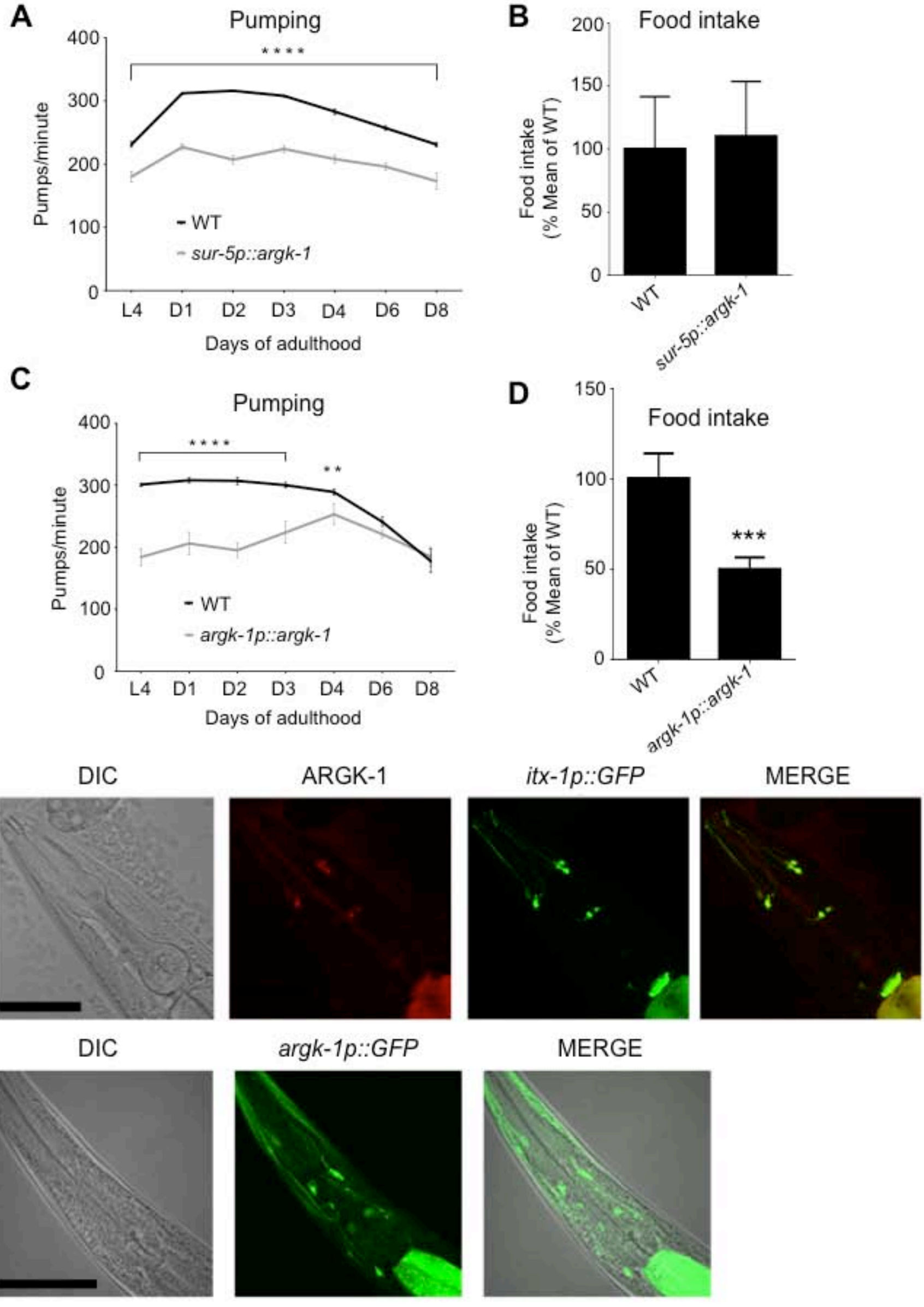
A
Pumping
Pumps/minute
400
300
200
100
0
****
WT
sur-5p::argk-1
L4
D1
D2
D3
D4
D6
D8
Days of adulthood
B
Food intake
Food intake (% Mean of WT)
200
150
100
50
0
WT
sur-5p::argk-1
C
Pumping
Pumps/minute
400
300
200
100
0
****
**
WT
argk-1p::argk-1
L4
D1
D2
D3
D4
D6
D8
Days of adulthood
D
Food intake
Food intake (% Mean of WT)
150
100
50
0
***
WT
argk-1p::argk-1
E
DIC
ARGK-1
itx-1p::GFP
MERGE
F
DIC
argk-1p::GFP
MERGE

Figure S3. Pharyngeal Pumping Phenotypes and Expression Patterns in Transgenic *C. elegans* Overexpressing ARGK-1; Related to Figure 3.

(**A**) Pharyngeal pumping rates were quantified at different time points (larval stage L4 and Day 1 [D1] to Day 8 [D8] of adulthood) in WT animals or transgenic animals expressing *sur-5p::argk-1::gfp.* N = 10 animals; error bars are SEM. ****p < 0.0001 by one-way ANOVA.

(**B**) Food intake was measured in transgenic animals expressing *sur-5p::argk-1::gfp* (Line A) and non-transgenic siblings (WT). N > 100 animals; error bars are SD. p = 0.91 by Student's *t*-test. The experiment was repeated twice with similar results.

(**C**) Pharyngeal pumping rates were quantified as in (**A**) in WT animals or transgenic animals expressing *argk-1p::argk-1::mcherry* (Line A). N = 10 animals; error bars are SEM. **p < 0.01, **** *P* < 0.0001 by one-way ANOVA. A separate strain (Line B) gave similar results (data not shown).

(**D**) Food intake was measured in the same strains as in (**C**) transgenic animals expressing *argk-1p::argk-1::mcherry* (Line A) and non-transgenic siblings (WT). N > 100 animals; error bars are SD. ****P* < 0.001 by Student's *t*-test. The experiment was repeated once with similar results. Food intake was measured in transgenic animals expressing *argk-1p::argk-1::mcherry* and in non-transgenic siblings (WT). N > 100 animals; error bars are SD. ***p < 0.001 by Student's *t*-test. The experiment was repeated once with similar results. A separate line (Line B) gave similar results (data not shown).

(**E**) Expression pattern of ARGK-1 (*argk-1p::argk-1::mCherry*) shows overlap with a transcriptional GFP-tagged reporter for the Neurexin superfamily member *itx-1*, which expresses in outer and inner labial socket cells (Haklai-Topper *et al.*, 2011). Overlap in fluorescence signals was seen in the head region of several of the double-extrachromosomal animals analyzed (F1 cross progeny from cross between *argk-1p::argk-1::mCherry* (Line A) and OH13790/*itx-1p::gfp*, n=7). The pharyngeal bulb is visible in the differential interference contrast image (DIC). Scale bar, 50 µm. Animals were raised at 20°C.

(**F**) Expression pattern of *argk-1* in the head region of Day-1 adult *C. elegans* expressing GFP from an endogenous promoter (*argk-1p::gfp*). This reporter shows expression in the same glia as the translational *argk-1p::argk-1::mCherry* reporter (data not shown), as well as in other cells including the intestine. Several independent transgenic strains showed similar expression patterns (data not shown). The pharyngeal bulb is visible in the differential interference contrast image (DIC). Scale bar, 50 µm. Animals were raised at 20°C.

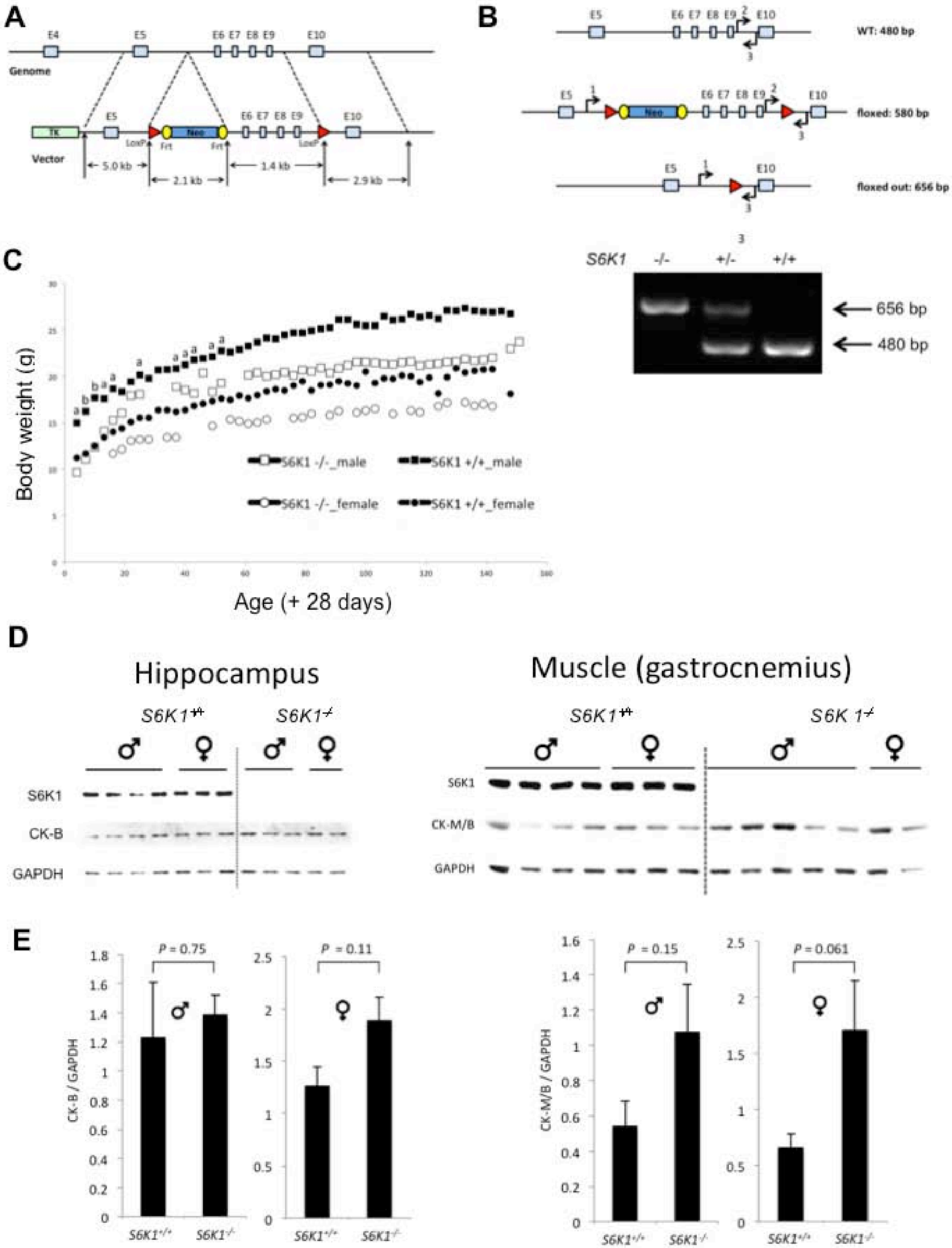
A
E4
E5
E6 E7 E8 E9
E10
Genome
TK
Neo
LoxP
Frt
Vector
5.0 kb
2.1 kb
1.4 kb
2.9 kb
B
WT: 480 bp
floxed: 580 bp
floxed out: 656 bp
S6K1 -/- +/- +/+
656 bp
480 bp
C
Body weight (g)
Age (+ 28 days)
S6K1 -/-_male
S6K1 +/+_male
S6K1 -/-_female
S6K1 +/+_female
D
Hippocampus
Muscle (gastrocnemius)
S6K1
CK-B
CK-M/B
GAPDH
E
P = 0.75
P = 0.11
P = 0.15
P = 0.061
CK-B / GAPDH
CK-M/B / GAPDH

Figure S4. Generation of S6K1 Knockout Mice, and Creatine Kinase Expression in Hippocampus and Skeletal Muscle of S6K1 Knockout Mice; Related to Figure 4.

(**A**) LoxP sites were introduced flanking exon 6 to exon 9 of the S6K1 gene. Floxed mice were bred to a Cre deleter strain expressing Cre recombinase to generate null alleles.

(**B**) Genotyping for *S6K1* alleles. Amplification products of wild-type (+/+), heterozygote (+/-), and homozygote (-/-) nulls following PCR with the primers indicated in the diagram and listed in the Supplemental Experimental Procedures.

(**C**) Plot of body weight versus age. Like other *S6K-/-* lines (Shima et al., 1998), the new strain had a mean body size smaller than control mice. Mice were weighed every other day for 5 months beginning at 4 weeks of age. Points represent the mean values of n = 8 *S6K1+/+* male mice; n = 3 *S6K1-/-* male mice; n = 5 *S6K1+/+* female mice; and n = 2 *S6K1-/-* female mice. Statistical significance between *S6K1+/+* and *S6K1-/-* mice was determined using an unpaired, two-tailed Student's *t*-test. a: $p < 0.05$, b: $p < 0.01$.

(**D**) Creatine kinase (CK) levels were assessed in *S6K1+/+* (n = 7) and *S6K1-/-* (n = 5-7) mice hippocampus (CK-B, brain isoform) and muscle (gastrocnemius, CK-M/B, myocardial/brain isoform) lysates. Males and females of age 5-8 weeks were analyzed.

(**E**) Relative CK levels (normalized to GADPH) from panels in (**D**) were quantified. Error bars, ± SEM. *P* values were derived from unpaired, two-tailed Student's *t*-test.

CK-M/B protein levels were also evaluated in the heart, but no difference was observed between WT and *S6K1-/-* mice (data not shown).

SUPPLEMENTAL TABLES

Tables S1-S3 (separate Excel files):

Table S1. List of Protein Spectral Counts in N2/wild-type and *rsks-1*/S6K Mutants; Related to Experimental Procedures.

Peptide counts detected in 2 biological repeats of day-1 N2-WT (wild-type) and *rsks-1*(sv33) mutants (_1 and _2); each of these samples were run twice (#1 and #2), and organized by Protein ID. ARGK-1 (F44G3.2) and AAK-2 (T01C8.1) are highlighted in blue. Spectral count fold changes and GO analyses are shown in **Tables S2, S3** in this document.

Table S2. Proteins Identified to be Relatively More Abundant in *rsks-1*/S6K Mutants; Related to Experimental Procedures.

(**A**) List of 139 proteins that were significantly more abundant in day-1 adult *rsks-1(sv31)* mutants compared to age-matched N2/wild-type animals. The spectral count (SpC) fold changes are shown in red, along with site of gene expression (www.wormbase.com). Two statistical approached were used to calculate p values: (1) a moderated *t*-test implemented in the LIMMA BioConductor package (primary *P* value, column E), and the Welch statistical test (secondary p value, column E), see Supplemental Experimental Procedures. Statistical significance was defined as $p \leq 0.05$, which corresponded to a ≥1.8-fold difference in SpC ratios.

(**B**) GO analysis of these proteins, see Supplemental Experimental Procedures.

Table S3. Proteins Identified to be Relatively Less Abundant in *rsks-1*/S6K Mutants; Related to Experimental Procedures.

(**A**) List of 200 proteins that were significantly less abundant in day-1 adult *rsks-1(sv31)* mutants compared to age-matched N2/wild-type animals. The spectral count (SpC) fold changes are shown in blue, along with site of gene expression (www.wormbase.com). Two statistical approached were used to calculate *P* values: (1) a moderated t-test implemented in the LIMMA BioConductor package (primary *P* value, column E), and the Welch statistical test (secondary *P* value, column E), see Supplemental Experimental Procedures. Statistical significance was defined as $P \leq 0.05$, which corresponded to a ≥1.8-fold difference in SpC ratios.

(**B**) GO analysis of these proteins, see Supplemental Experimental Procedures.

Table S4. Survival Analysis of *rsks-1*/S6K and *argk-1* Single or Double Mutants.

Trial	Strain	% alive ± SEM	p value vs WT	p value vs *rsks-1*
Thermotolerance assay				
1	WT	7±3		<0.0001
	rsks-1	45±5	<0.0001	
	argk-1	4±2	n.s	<0.0001
	rsks-1; argk-1	24±5	<0.05	<0.01
2	WT	6±2		<0.0001
	rsks-1	63±5	<0.0001	
	argk-1	3±2	n.s	<0.0001
	rsks-1; argk-1	29±5	<0.001	<0.001
3	WT	6±3		<0.0001
	rsks-1	40±5	<0.0001	
	argk-1	5±2	n.s	<0.0001
	rsks-1; argk-1	19±0	n.s	<0.001
Thermorecovery assay				
1	WT	1±1		<0.0001
	rsks-1	70±5	<0.0001	
	argk-1	6±2	n.s	<0.0001
	rsks-1; argk-1	46±6	<0.0001	<0.001
2	WT	8±6		<0.0001
	rsks-1	80±1	<0.0001	
	argk-1	23±4	n.s	<0.0001
	rsks-1; argk-1	63±5	<0.0001	<0.05
3	WT	0±0		<0.0001
	rsks-1	47±8	<0.0001	
	argk-1	3±1	n.s	<0.0001
	rsks-1; argk-1	26±4	<0.001	<0.01

Table S4. Survival Analysis of *rsks-1*/S6K and *argk-1* Single or Double Mutants; Related to Experimental Procedures.

Thermotolerance analyses of wild-type (WT, N2-Tuck), *rsks-1(sv31)*, *argk-1(ok2993),* and *rsks-1(sv31); argk-1(ok2993)* animals fed OP50 bacteria. Animals were raised at 20ºC until day 3 of adulthood and then subjected to two different paradigms for assessing resistance to heat stress. In the thermotolerance test, animals (N = 80) were subjected to acute thermal stress at 35°C for 8 h and survival was then scored (top of table). In the thermorecovery test, animals were subjected to thermal stress at 35°C for 6 h and then shifted to 20ºC for 20 h of recovery, after which survival was scored (bottom of table). The table is organized so that experiments carried out in parallel are boxed, and the independent trials are numbered. Data are presented as survival (% alive ± SEM), with p values compared to WT animals or to *rsks-1(sv31)* mutants (calculated by one-way ANOVA).

Table S5. Lifespan Analysis of *C. elegans* with Reduced *argk-1* Levels.

A. Lifespan analysis of *argk-1* single- or double mutants						
Trial	**Strains**	**Average lifespan (days)**	**% Lifespan extension compared to WT**	**Number of events**	**p value vs WT**	**p value vs *rsks-1***
Experiments using *rsks-1(sv33)*; *argk-1(ok2993)*						
1*	WT	17.5	-	73/100	-	<0.0001
	rsks-1	22.5	29%	60/100	<0.0001	-
	argk-1	17.2	-2%	73/100	0.86	<0.0001
	rsks-1; argk-1	17.6	0%	44/100	0.95	<0.0001
2	WT	19.1	-	75/100	-	<0.0001
	rsks-1	25.0	31%	78/100	<0.0001	-
	argk-1	20.3	6%	64/100	0.38	<0.0001
	rsks-1; argk-1	19.0	0%	20/100	0.63	<0.0001
3	WT	19.9	-	83/100	-	<0.0001
	rsks-1	25.2	27%	93/100	<0.0001	-
	argk-1	19.1	-4%	71/100	0.15	<0.0001
	rsks-1; argk-1	20.0	0%	80/100	0.14	<0.0001
Experiments using *rsks-1(sv33); argk-1(ok2973)*						
1	WT	21.6	-	82/100	-	0.0009
	rsks-1	23.1	7%	68/100	0.0009	-
	argk-1	19.9	-8%	87/100	0.0010	<0.0001
	rsks-1; argk-1#	18.1	-16%	55/100	0.0044	<0.0001
2	WT	18.7	-	95/100	-	<0.0001
	rsks-1	21.7	16%	59/100	<0.0001	-
	argk-1	18.8	0%	93/100	0.95	<0.0001
	rsks-1; argk-1#	18.3	0%	58/100	0.93	0.0002
3	WT	19.7	-	85/100	-	<0.0001
	rsks-1	22.3	13%	61/100	<0.0001	-
	argk-1	20.0	0%	90/100	0.27	0.0016
	rsks-1; argk-1#	16.8	-15%	68/100	0.018	<0.0001
Experiments using both *rsks-1(sv33)*; *argk-1*(*ok2993)* and *rsks-1(sv33)*; *argk-1*(*ok2973)*						
1	WT	18.8	-	86/100	-	<0.0001
	rsks-1	23.8	27%	78/100	<0.0001	-
	argk-1(ok2993)	17.9	-5%	82/100	0.18	<0.0001
	rsks-1; argk-1$^{\#}$	20.0	6%	81/100	0.0017	<0.0001
	argk-1(ok2973)	16.4	-13%	82/100	0.17	<0.0001
	rsks-1; argk-1'	19.7	5%	77/100	0.0092	0.046

Table S5 (Continued):

A. Lifespan analysis of *argk-1* single- or double mutants						
Trial	**Strains**	**Average lifespan (days)**	**% Lifespan extension compared to WT**	**Number of events**	**p value vs WT**	**p value vs *rsks-1***
Experiments using *daf-2(e1370); argk-1*(*ok2993)* and *eat-2(ad1116); argk-1*(*ok2993)* mutants						
1	WT	15.7	-	41/93	-	-
	argk-1	13.1	-17%	39/96	0.01	-
	daf-2	35.7	127%	60/96	<0.0001	-
	eat-2	23.2	48%	43/93	<0.0001	-
	daf-2; argk-1	40.8	160%	65/96	<0.0001	0.16
	eat-2; argk-1	24.6	57%	25/84	<0.0001	0.42
2	*daf-2*	39.8	-	85/103	-	-
	daf-2; argk-1	38.1	-	87/104	-	0.32
2'	*eat-2*	24.9	-	49/83	-	-
	eat-2; argk-1	27.8	-	35/98	-	0.07

B. Lifespan analysis of longevity mutants subjected to *argk-1* RNAi during adulthood							
Trial	**Strains**	***argk-1* RNAi Average Lifespan (days)**	**Number of events**	**Control RNAi Average Lifespan (days)**	**Number of events**	**% Lifespan extension on *argk-1* RNAi**	**p value vs control**
1*	WT	21.6	86/100	21.2	88/100	0%	0.32
2		19.5	94/100	19.7	95/100	0%	0.66
3		16.4	85/100	16.8	84/100	0%	0.40
1*	*rsks-1*	22.2	70/100	26.2	61/100	-15%	<0.0001
2		19.1	61/100	21.4	48/100	-11%	0.04
3		17.9	71/100	20.5	65/100	-13%	<0.0008
1*	*daf-2*	30.6	95/100	30.6	74/100	0%	0.63
2		28.5	90/100	28.8	96/100	0%	0.72
3		27.5	85/100	27.6	57/80	0%	0.80
1*	*eat-2*	25.6	66/100	25.3	82/100	0%	0.67
2		26.1	60/100	25.5	57/100	0%	0.60
3		25.8	53/100	25.7	56/100	0%	0.83
1*	*clk-1*	27.2	86/100	28.2	83/100	0%	0.05
2		25.8	86/100	25.7	88/100	0%	0.77
3		27.2	88/100	26.7	91/100	0%	0.42

Table S5. Lifespan Analysis of *C. elegans* with Reduced *argk-1* Levels; Related to Figure 1.

(**A**) Lifespan analyses of wild-type (WT, N2-Tuck), *rsks-1(sv31)*, *argk-1(ok2973), argk-1(ok2973), daf-2(e1370), and eat-2(ad1116)* single mutants, *rsks-1(sv31)*; *argk-1(ok2993), daf-2(e1370); argk-1(ok2993),* and *eat-2(ad1116); argk-1(ad2993)* double mutants. All experiments were carried out at 20°C and on OP50 bacteria. The table is organized so that lifespan experiments carried out in parallel

are boxed, and individual trials are numbered. Number of events indicates the number of dead animals/total number of animals analyzed). *P* values were calculated using the Mantel-Cox log-rank test. # indicates *ok2993* allele and **'** indicates *ok2973* allele. ^, p value between *rsks-1*; *argk-1* and *rsks-1*; *argk-1* double mutants overexpressing ARGK-1 from endogenous promoter. *, data shown in **Figure 1A**.

(**B**) Lifespan analyses of wild-type (WT, N2-Tuck), *rsks-1(sv31)*, *daf-2(mu150)*, *eat-2(ad1116),* and *clk-1(e2519)* animals fed bacteria containing empty vector (control) or expressing *argk-1* dsRNA during adulthood at 20ºC. *daf-2(mu150)* mutants were maintained at 15ºC, the eggs were hatched and left for 1 day at 15°C, and then animals were transferred to 25ºC until day 1 of adulthood. The table is organized so that different trials with specific strains are numbered and boxed, and experiments carried out in parallel are in the same order. Data show the average lifespan, the number of events (number of dead animals/total number of animals analyzed), the percent lifespan extension of animals subjected to *argk-1* RNAi compared to control bacteria, and the p value for this comparison. *P* values were calculated using the Mantel-Cox log-rank test. *, data shown in **Figures 1B-1E**.

Table S6. Lifespan Analysis of *C. elegans* Overexpressing ARGK-1.

A. Lifespan analysis of transgenic strains with ubiquitous *sur-5* promoter and fed OP50 bacteria							
Trial	**WT background**	**Average lifespan (days)**	**Number of events**	**Control average lifespan (days)**	**Control number of events**	**Δ avg (%)**	**p value vs control**
1*	*sqEx27[sur-5p::argk-1::gfp]* Line A (25°C)	12.6	89/100	10.4	96/100	21%	<0.0001
2		12.1	90/100	10.0	72/100	21%	<0.0001
1	*sqEx27[sur-5p::argk-1::gfp]* Line A (20°C)	20.1	52/100	17.5	73/100	15%	<0.0001
2		20.1	55/100	17.3	100/100	16%	<0.0001
3		19.8	80/104	15.8	81/105	25%	<0.0001
1	*sqEx28[sur-5p::argk-1::gfp]* Line B (20°C)	19.0	82/100	16.1	85/100	18%	<0.0001
2		20.1	46/55	17.3	87/100	16%	0.0002
1	*sqIs32[sur-5p::argk-1::gfp]* (20°C)	25.3	116/126	22.2	117/123	14%	<0.0001
2		24.5	92/100	22.4	88/100	9%	0.0075
Trial	***rsks-1* background**	**Average lifespan (days)**	**Number of events**	**Control average lifespan (days)**	**Control number of events**	**Δ avg (%)**	**p value vs control**
1	*rsks-1; sqEx27 [sur-5p::argk-1::gfp]* (25°C)	13.0	91/100	12.9	90/100	0%	0.75
2		12.7	92/100	13.4	95/100	1%	0.08
Trial	***aak-2* background**	**Average lifespan (days)**	**Number of events**	**Control average lifespan (days)**	**Control number of events**	**Δ avg (%)**	**p value vs control**
1*	*aak-2; sqEx27 [sur-5p::argk-1::gfp]* (20°C)	15.6	85/100	22.4	41/100	-30%	<0.0001
2		15.7	78/100	19.9	57/100	-21%	0.0005
1	*aak-2; sqEx27 [sur-5p::argk-1::gfp]* (25°C)	8.9	99/100	9.7	100/100	-8%	0.004
2		9.3	80/100	10.4	93/100	-11%	0.0002

Table S6 (continued):

B. Lifespan analysis of transgenic strains with ubiquitous *sur-5* promoter on *pha-4* RNAi bacteria							
Trial	**Strains**	**Average lifespan on *pha-4* RNAi (days)**	**Number of events**	**Average lifespan on control RNAi (days)**	**Number of events**	**Δ avg (%)**	**p value vs control**
1	WT	11.6	61/87	13.5	74/98	-14%	0.004
	sqEx27[sur-5p::argk-1::gfp] (20°C)	13.9	79/97	16.4	74/85	-15%	0.003
2	WT	8.9	99/100	8.9	96/100	0%	0.72
	sqEx27[sur-5p::argk-1::gfp] (25°C)	8.2	100/100	9.9	96/100	-17%	<0.0001
3	WT	9.0	99/100	8.8	98/100	0%	0.98
	sqEx27[sur-5p::argk-1::gfp] (25°C)	8.3	100/100	9.7	99/100	-14%	<0.0001

C. Lifespan analysis of transgenic strains with endogenous *argk-1* promoter fed OP50 bacteria							
Trial	**WT background**	**Average lifespan (days)**	**Number of events**	**Control average lifespan (days)**	**Control number of events**	**Δ avg (%)**	**p value vs control**
1	*sqEx09[argk-1p::argk-1::mCherry]* Line A (25°C)	10.6	92/100	9.0	89/100	18%	<0.0001
2		10.4	90/100	8.5	89/100	22%	<0.0001
3		12.8	93/100	10.6	88/100	21%	<0.0001
1	*sqEx09[argk-1p::argk-1::mCherry]* Line A (20°C)	20.3	71/90	15.5	90/100	31%	<0.0001
2		20.7	63/80	17.4	60/79	19%	0.0002
1	*sqEx10[argk-1p::argk-1::mCherry]* Line B (25°C)	10.7	87/100	9.8	86/100	9%	0.062
2		9.6	93/100	8.9	92/100	8%	0.066
Trial	***rsks-1* background**	**Average lifespan (days)**	**Number of events**	**Control average lifespan (days)**	**Control number of events**	**Δ avg (%)**	**p value vs control**
1	*sqEx09[argk-1p::argk-1::mCherry]* (25°C)	10.4	89/100	10.4	94/100	0%	0.51
2		10.4	92/100	10.8	91/100	0%	0.48

Table S6. Lifespan Analysis of *C. elegans* Overexpressing ARGK-1; Related to Figure 3A and 3B.

(**A**) Lifespan analyses of wild-type (WT), *rsks-1(sv31),* or *aak-2(ok524)* animals carrying extrachromosomal or integrated arrays expressing *sur-5p::argk-1::gfp* (and *rol-6* co-injection marker, which does not affect longevity (Lin et al., 2001)) compared with non-transgenic siblings fed OP50 bacteria. Two different non-integrated transgenes (Line A and B) and one integrated transgenic strain were analyzed in two independent experiments for each strain. Lifespan analyses were carried out at 20°C and 25°C as labeled. The table is organized so that different trials are numbered and boxed. Data

show the average lifespans of ARGK-1-overexpressing or control animals, the percent lifespan extension compared to control animals (Δ avg), the number of events (number of dead animals/total number of animals analyzed), and the p value compared to control animals. p values were calculated using the Mantel-Cox log-rank test. *, data shown in **Figures 3A-3B**.

(**B**) Lifespan analyses of transgenic animals carrying extrachromosomal arrays expressing *sur-5p::argk-1::gfp* compared with non-transgenic siblings fed HT115 bacteria containing empty vector (control), or expressing *gfp* (negative control) or *pha-4* dsRNA. WT (N2-Tuck) animals were also used for some *pha-4* RNAi experiments. Animals were raised on OP50 bacteria until they reached day 1 of adulthood and were then transferred to plates containing control or dsRNA-expressing bacteria. Lifespan analyses were carried out at 20°C or 25°C as labeled. The table is organized so that different trials are numbered and boxed. Data show the average lifespan, the percent lifespan extension compared to control animals (Δ avg), the number of events (number of dead animals/total number of animals analyzed), and the p value compared to control animals.

(**C**) Lifespan analyses of transgenic wild-type (WT) and *rsks-1(sv31)* animals carrying extrachromosomal arrays expressing *argk-1p::argk-1::gfp* (and *rol-6* co-injection marker, which does not affect longevity (Lin et al., 2001)) compared with non-transgenic siblings fed OP50 bacteria. Two different non-integrated lines (A and B) and one integrated transgenic strain were analyzed in two independent experiments for each strain. Lifespan analyses were carried out at 20°C or 25°C as labeled. The table is organized so that different trials are numbered and boxed. Data show the average lifespan, the percent lifespan extension compared to control animals (Δ avg), the number of events (number of dead animals/total number of animals analyzed), and the p value compared to control animals. p values were calculated using the Mantel-Cox log-rank test.

Table S7. *C. elegans* Strains Used in This Study.

Name	Genotype	Strain obtained from
Published strains used in this study		**Comments**
N2-Hansen	Wild-type (WT)	Hansen lab (N2-MH), from Kenyon lab
N2 Tuck	Wild-type (WT)	Tuck lab (N2-T)
CB4876	*clk-1(e2519) III*	Kenyon lab
CF1844	*fer-15(b26) II; daf-2(mu150) III; fem-1(hc17) IV*	Kenyon lab
CF1908	*eat-2(ad1116) II*	Kenyon lab
GA1001	*aak-2(ok524) X*	Hubbard lab
OH13790	*otEx58[itx-1p::gfp + rol-6]*	Hobert lab
LX1161	*lin-15(n765) X; vsEx518[kcc-3p::gfp::kcc-3* (3'UTR) *+ lin-15(+)]*	Shaham lab
RB2193	*argk-1(2973) V*	CGC[1]
RB2211	*argk-1(2993) V*	CGC
VB633	*rsks-1 (sv31) III*	Tuck lab
WBM59	*uthIs272(myo-2p::tdTomato)*	Mair lab
WBM60	*uthIs248[aak-2(1-321)::gfp::unc-54 UTR + myo-2p::tdTomato]*	Mair lab
New strains created for this study		**Comments**
MAH145	*sqEx04 [atg-18p::atg-18::gfp + rol-6(su1006)]*	pMH841 inj. into N2-MH
MAH160	*sqEx09[argk-1p::argk-1::mCherry+ rol-6(su1006)]* (Line A)	pMH826 inj. into N2-T
MAH161	*sqEx10[argk-1p::argk-1::mCherry+ rol-6(su1006)]* (Line B)	pMH826 inj. into N2-T
MAH164	*rsks-1(sv31) III*; *sqEx09[argk-1p::argk-1::mCherry + rol-6(su1006)]*	
MAH172	*argk-1(ok2973) V*	RB2193 4x outcrossed to N2-MH, 4x outcrossed to N2-Tuck (N2-T)
MAH174	*rsks-1(sv31) III; argk-1(ok2973) V*	
MAH175	*rsks-1(sv31) III; argk-1(ok2993) V*	
MAH205	*argk-1(ok2993) V*	RB2211 4x outcrossed to N2-MH, then 4x outcrossed to N2-T
MAH247	*sqIs25[atg-18p::atg-18::gfp + rol-6(su1006)]*	MAH145 integrated and 4x outcrossed to N2-MH
MAH258	*rsks-1(sv31) III; sqEx27[sur-5p::argk-1::gfp + rol-6(su1006)]*	pMH929 inj. into VB633
MAH264	*sqEx27(sur-5p::argk-1::gfp + rol-6(su1006)]* (Line A)	MAH258 crossed to N2-T
MAH275	*rsks-1(sv31) III; sqIs25[atg-18p::atg-18::gfp + rol-6(su1006)]*	
MAH276	*sqEx28[sur-5p::argk-1::gfp + rol-6(su1006)]* (Line B)	pMH929 inj. into N2-T
MAH286	*aak-2(ok524) X; sqEx27[sur-5p::argk-1::gfp + rol-6(su1006)]*	
MAH300	*sqIs32[sur-5p::argk-1::gfp + rol-6(su1006)]*	MAH264 integrated and 4x outcrossed to N2T
MAH312	*lin-15(n765)? X; vsEx518[kcc-3p::gfp::kcc-3* 3'UTR *+lin-15(+)]*; *sqEx09[argk-1p::argk-1::mCherry+ rol-6(su1006)]*	MAH160 crossed to LX1161

Table S5 (Continued):

Name	Genotype	Strain obtained from
New strains created for this study		**Comments**
MAH412	*eat-2(ad1116) II; argk-1(ok2993) V*	
MAH413	*daf-2(e1370) III; argk-1(ok2993) V*	
MAH547	*sqEx82[argk-1p::gfp + rol-6(su1006)]*	pMH899 inj. into N2-MH

Table S7. *C. elegans* Strains Used in This Study; Related to Experimental Procedures.

[1]: *Caenorhabditis* Genetics Center (CGC), both strains carry deletions of regions important for enzyme activity and substrate binding (data not shown).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

C. elegans and Bacterial Strains

*C. elegans s*trains (**Table S7**) were maintained on OP50 *E. coli* at 20°C or 25°C as described (Brenner, 1974), except that animals were grown on HT115 bacteria for RNA interference (RNAi) experiments. For those experiments, an *argk-1* RNAi clone from the Vidal library (Rual et al., 2004) was used, and bacteria were grown as previously described (Hansen et al., 2005).

Construction of Transgenic Strains

DNA constructs for transgenesis were made using the Gateway Technology protocol (Hartley et al., 2000). For *argk-1* constructs, PCR products were generated for the *C. elegans argk-1*/F44G3.2 promoter sequence using primers: Fwd 5' GGG GAC AAC TTT GTA TAG AAA AGT TGA ACT ATT TTT CCC TTC GTG TTT 3' and Rvs 5' GGG GAC TGC TTT TTT GTA CAA ACT TGT CAT GCC AAG ACA CAG AGT TGA 3' (1.2 kb) and for *argk-1* cDNA using primers: Fwd 5' GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GTC ATC TCA AAT TCG CGG CT 3' and Rvs 5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TGT TTC TTT CTC CTC ATC TC 3' (1.1 kb). These PCR products were sequenced and introduced into Gateway entry vectors, which were used to generate the two translational reporters pMH826/*argk-1p::argk-1::mCherry* and pMH929/*sur-5p::argk-1::gfp* (*sur-5* promoter Gateway entry clone was a kind gift from Dr. Supriya Srinivasan), and a transcriptional reporter pMH899/*argk-1p::gfp*. For *atg-18* constructs, PCR products were generated for the *C. elegans atg-18/*F41E6.13 promoter sequence using primers: Fwd 5' GCA TGC GGA AAA TCG CAC TGC GCC ATT CGG ACC 3' and Rvs 5' ATC TGT AAA AGT TTA AAT GTA TTT TTA AAG GGG GAC TGC TTT TTT GTA CAA ACT TGT C 3' (540 bp) and for *atg-18* cDNA using primers: Fwd 5' GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GTC GGC TAC AAC ATC AGA A 3' and Rvs 5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA GCC GCT GGT GTG GCT CAT T 3' (1.6 kb). These PCR products were sequenced and introduced into Gateway entry vectors, which were used to generate the translation reporter pMH841*/atg-18p::atg-18::gfp.*

For transgenesis, plasmid DNA was prepared using QIAPrep MiniPrep kit (Qiagen). N2-Tuck animals were injected with 10 ng/µl of pMH826/*argk-1p::argk-1::mCherry* combined with 90 ng/µl of pRF4/*rol-6* co-injection marker; VB633/*rsks-1(sv31)* animals were injected with 50 ng/µl of pMH929/ *sur-5p::argk-1::gfp* combined with 50 ng/µl of pRF4/*rol-6*; and N2-Hansen animals were injected with 10 ng/ul of pMH899/*argk-1p::gfp* combined with 90 ng/µl of pRF4/*rol-6,* or 22 ng/µl of pMH841*/ atg-18p::atg-18::gfp* combined with 20 ng/µl of pRF4/*rol-6* (see **Table S7** for strain information), as previously described to create extrachromosomal arrays (Berkowitz et al., 2008). Integration of extrachromosomal arrays was performed by γ-irradiation followed by outcrossing four times.

The *rsks-1(sv31)* mutation was introduced into the ATG-18::GFP expressing strain, and expression levels were compared using a Leica MZ16F microscope configured with a Leica DFC310FX camera (**Figure S1**).

Two-Dimensional Liquid Chromatography-Tandem Mass Spectrometry (2DLC-MS/MS)

<u>*C. elegans* protein extraction and digestion</u>

A previously published protocol for protein extraction for LC-MS/MS analysis (Brill et al., 2009) was adapted for *C. elegans* use. Duplicate samples of ~3×10^4 day 1 wild-type (N2-Hansen) or VB633/ *rsks-1(sv31)* animals were collected in M9 media. Proteins were extracted by resuspension of worms in ice-cold urea lysis buffer containing protease inhibitors (5 µg/µl aprotinin, 10 µg/µl leupeptin, 10 µg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride [PMSF]) and phosphatase inhibitor cocktail I (Sigma-Aldrich P#P2850) and homogenization using 0.5 mm Zirconia beads in a Fastprep cell disrupter (Savant Instruments, Holbrook, NY). Protein concentrations were determined by Bradford Assays (Bio-Rad, Hercules, CA). Bovine Serum Albumin (BSA) was added at 1 nmol/mg and β-casein was added at 1, 2, 3, and 4 nmol/mg C. elegans protein, respectively, to individual lysates as internal standards. The samples were incubated at room temperature for 30 min, N,N-dimethylacrylamide (Sigma-Aldrich P#274135) was added to a final concentration of 0.5% v/v, and the samples were again incubated at room temperature for 30 min. Dithiothreitol was added to a final concentration of 20 mM to quench the

reaction. Lysates were centrifuged for 15 min at 14,000 g at room temperature and the pellets were discarded. Samples of 2 mg protein were precipitated using methanol-chloroform extraction with 4 sample volumes of methanol and 1 sample volume of chloroform as described previously (Brill et al., 2009). Three sample volumes of water were then added, and the samples were centrifuged for 25 min at 4,000 g at room temperature. The upper aqueous layer was discarded, 4 sample volumes of methanol were added, and the tubes were vortexed and centrifuged for 25 min at 4,000 g at room temperature. The supernatant was discarded, and the dried protein pellet was stored at -80ºC.

Trypsin digest and desalting

Protein pellets (2 mg) were solubilized in 50 mm $(NH_4)_2HCO_3$ by vortexing and sonication. Each sample was mixed with 40 µg of sequencing grade trypsin (Promega) and the samples were shaken at 600 rpm on a Thermomixer (Eppendorf) overnight at 37°C. Peptides were desalted with a C18 cartridge as described previously (Brill et al., 2009), dried overnight at 35ºC using a SpeedVac (Thermo Fisher Scientific), and stored at -80 ºC.

2DLC-MS/MS total proteome analysis

These procedures were as described previously (Brill et al., 2009). In brief, peptides were separated by strong cation exchange chromatography (SCX). Peptides from the SCX fractions were separated by reverse-phase (RP) LC, using a Paradigm HPLC, a Magic C18 column (Michrom), and a gradient from 2.0% (minute 0.0) to 5.0% (minute 2.0) solvent B (solvent A = 0.1% formic acid in water, solvent B = 100% acetonitrile), to 35.0% solvent B at 180.0 min, to 80% solvent B from 180.1 to 186.0 min, and to 2.0% solvent B from 186.1 to 196.1 min. The RPLC was coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher) via an ADVANCE electrospray ionization source (Michrom). Precursor ions (m/z from 300 to 2000) were scanned in the Orbitrap at a resolution of 60,000, and MS/MS scanning consisted of fragmenting the 4 most abundant precursor ions by collision-induced dissociation and scanning the product ions in the linear ion trap. Charge state screening and monoisotopic precursor selection and dynamic exclusion for 120 sec with a repeat count of 2 was enabled. Each of the two biological replicates was analyzed by 2DLC-MS/MS twice, and peptides from ~60% of *C. elegans'* predicted proteins were identified (~10,000 of ~18,000 proteins, **Table S1**). The raw MS/MS data was deposited in Peptide Atlas under the accession number: PASS00820.

MS/MS spectra were searched against the Wormpep201 database (European Bioinformatics Institute) using Sorcerer™-SEQUEST® (SageN Research). Differential oxidation of Met residues, and precursor ion mass tolerance of 5 ppm maximum error were specified. Search results were filtered at a false discovery rate of <0.02 (ProteinProphet, Trans-Proteomic Pipeline). Label-free quantification was by spectral counting (Liu et al., 2004). At least two different peptides per protein were required for positive identification.

Bioinformatics

The list of MS spectral count data was normalized by the quantile approach (Bolstad et al., 2003), and missing values were imputed using an offset method. Differential expression was assessed using two statistical approaches performed by two bioinformatics groups at the Sanford Burnham Prebys Medical Discovery Institute: (1) a moderated *t*-test implemented in the LIMMA BioConductor package (Smyth, 2004) with *P* values adjusted by the Benjamin-Hochberg approach (Hochberg and Banjamin, 1995) (performed by Dr. Jian-Liang Li, Orlando, FL), and (2) the Welch statistical test (performed by Dr. Roy Williams, La Jolla, CA). The statistical significance criteria were defined as 1.8-fold difference in expression levels with *P* values less than or equal to 0.05. Proteins with significant differential expression from each of the two analyses were combined and a total of 339 proteins were obtained (**Tables S2, S3**).

Measurement of Body and Brood Sizes

To measure body size, 8–10 day-1 adult animals were mounted on a glass slide and photographed using a Leica MZ16F microscope configured with a Leica DFC310FX camera. Images were processed with Leica Application Suite analysis software and the body lengths of individual animals were measured using Image J software (**Figure S2**). Statistical significance was determined using one-way ANOVA. Unless noted, all statistical analyses were performed with GraphPad Prism software.

To measure brood size, 10 day-1 adult animals were allowed to lay eggs on media plates, with transfer to fresh plates every day. The plates were subsequently incubated for 2–3 days and the hatched progeny was counted. Statistical significance was determined using one-way ANOVA.

Heat-Stress Assays

Two different heat-stress assays were employed; thermotolerance and thermorecovery (**Table S4**). The thermotolerance assay was performed as previously described (Hansen et al., 2007); synchronized day-1 adult animals were shifted from 20°C to 35°C for 8 h and survival was scored. For the thermorecovery assay, the animals were placed on OP50 bacteria plates at 35°C for 6 h and then shifted back to 20°C for 24 h of recovery before scoring, as described previously (Kumsta et al., 2014). Statistical significance was determined using one-way ANOVA.

Confocal Imaging

At least 10 day-1 adult animals were raised at 20°C and mounted on a 2% agarose pad containing 15 mM NaN_3. Animals were imaged using a LSM Zeiss 710 scanning confocal microscope, and Z stacks were taken at 0.6 µm slices. mCherry excitation/emission wavelengths were 572/696 nm. GFP excitation/emission was limited to 493/523 nm to eliminate background auto-fluorescence.

Food-Intake Assay

Food intake was measured as described (Gomez-Amaro et al., 2015). Briefly, the assay was performed in liquid medium (S-complete medium with 50 µg/ml carbenicillin and 0.1 µg/ml fungizone) in flat-bottomed black optically clear 96-well plates (Costar). For each strain, freshly prepared *E. coli* OP50 (6 mg/ml) and 6–12 nematodes were added to each of 9 or 10 wells. Age-synchronized nematodes were seeded as L4 larvae and the plates were sealed with tape (Nunc) to prevent evaporation. To prevent self-fertilization, 5-fluor-2′-deoxyuridine (0.12 mM final; Sigma) was added at seeding. The absorbance of each well at 600 nm (OD600) was measured with a microplate reader (TECAN) on day 1 and day 4 of adulthood. The fraction of animals alive in each well was scored microscopically on the basis of movement on day 4 of adulthood. Food intake was defined as the difference in OD600 between day 1 and 4 ($OD_{day4} - OD_{day1}$). Data were normalized for worm number per well prior to analysis (**Figure S3**). A detailed protocol of the food-intake assay can be found at Nature Protocol Exchange (Petrascheck Lab Group). Data were statistically analyzed with t-tests using GraphPad Prism software.

Western Blot Analysis

Total protein was extracted from 60 (handpicked) day-1 adult animals grown on OP50 bacteria at 20ºC. Animals were washed thoroughly in M9 buffer and the worm pellets were lysed in 30 µl 2× SDS sample buffer. Half of each extract was separated on a 4–20% pre-cast SDS-PAGE gel (Invitrogen) and proteins were transferred to a PVDF membrane (Millipore). Immunoblotting was performed using antibodies to β-actin (Millipore MAB1501R, diluted 1:1000), phospho-AMPKα Thr 172 (Cell Signaling Technologies #2535, diluted 1:300) (to detect *C. elegans* AAK-2 (Selman et al., 2009), and phospho-ACC Ser 79 (Cell Signaling Technologies #3661, diluted 1:1000) (to likely detect *C. elegans* POD-2/W09B6.1: Three predicted POD-2 isoforms exist (a and b: both ~200 kDa, c: ~80 kD). We observed ~95 kDa, ~130 kDa, and >170 kDa bands in Western blots using this antibody; all of these bands were subject to similar regulation (data not shown). We have chosen to show/quantify the >170 kDa band because this size band is similar to predicted POD-2 isoform(s)). Immunoblots were developed by enhanced chemiluminescence (Pierce) and quantified using ImageJ software. Statistical significance was determined using one-way ANOVA.

Quantitative RT-PCR

Total RNA was isolated from a synchronized population of ~2000 nematodes on day 1 of adulthood raised on OP50 bacteria. After harvest, the animals were flash frozen in liquid nitrogen. RNA was extracted with Trizol (Life Technologies) and purified using a Qiagen RNeasy kit, with an additional DNA digestion step (Qiagen DNase I kit). Reverse transcription (1 µg per sample) was performed using M-MuLV reverse transcriptase and random 9-mer primers (New England Biolabs, Ipswich, MA) (Taubert et

al., 2006). Quantitative PCR was performed using SYBR Green Master Mix in an LC480 LightCycler (Roche, Basel, Switzerland) at an annealing temperature of 58°C and 40 cycles. The number of cycles was increased to 60 for the analysis of *argk-1*, because of the low abundance of this transcript in WT animals; the Crossing point (Cp) value for *argk-1* in WT animals was 30.8, compared to 19.0 in animals ubiquitously overexpressing ARGK-1.

A standard curve was obtained for each primer set by serially diluting a mixture of different cDNAs, and the standard curves were used to convert the observed CT values to relative values. mRNA levels of target genes were normalized to four housekeeping genes: large subunit of RNA polymerase II *ama-1*, nuclear hormone receptor *nhr-23*, cyclophilin *cyn-1*, and the putative ABC transporter *pmp-3* (Hoogewijs et al., 2008; Lapierre et al., 2013). Primer sequences are available upon request. The transcript levels of each strain were analyzed in three biological samples and ran with 2-3 technical replicates per biological sample (**Figure S1**). The average and standard error of the mean of every mRNA was calculated and normalized to WT levels. Statistical difference between each strain and the WT was calculated with Student's *t*-test (GraphPad Prism, La Jolla, CA).

Mouse Experiments

A conditional S6K1 knockout ($S6K1^{flox/flox}$) was generated by inserting lox P sites in the introns flanking exon 6 to exon 9. To generate null alleles, $S6K1^{flox/flox}$ mice were crossed to a Cre deleter strain expressing Cre recombinase. Mice were genotyped by PCR using primers flanking the lox P sites as indicated in **Figure S4**. $S6K1^{+/+}$ and $S6K1^{-/-}$ mice were generated by mating $S6K1^{+/-}$ mice on the C57BL/6 genetic background. PCR primers were: S6K1-WT forward: 5′-AGCCAGTATTGCAGTGCTTTGTGC-3′, S6K1-KO forward: 5′-GCTCAGCAGTTAAAGAGTACCGAC-3′, and S6K1 reverse: 5′-TGGCACAGGTTGTTGCCACAATGA-3′. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at the Buck Institute for Research on Aging.

Cerebellums, hippocampi and skeletal muscle (gastrocnemius) were harvested from 5–8-week-old littermates and immediately frozen in liquid nitrogen. Tissue sections were homogenized using the Omni TH homogenizer (Omni International, Kennesaw, GA) on ice in RIPA buffer (300 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0], protease inhibitor cocktail [Roche], and phosphatase inhibitor 2, 3 [Sigma]) and then centrifuged at 13,200 rpm for 10 min at 4°C. The supernatants were collected and protein concentrations were determined using the DC protein assay (Bio-Rad). Equal amounts of protein were resolved by SDS-PAGE (4–12% gel), transferred to membranes, and analyzed by Western blotting with protein-specific antibodies. Anti-S6K1 (#2708) and anti-GAPDH (#2118) were from Cell Signaling Technology, and anti-creatine kinase B (CK-B; sc-15157), anti-muscle tissue-specific isoform of creatine kinase (CK-M/B; sc-28898) antibodies were was from Santa Cruz Biotechnology. Protein bands were revealed using the Amersham ECL detection system (GE Healthcare) and quantified by densitometry using ImageJ software. Equal protein loading was verified by blotting for GAPDH. Statistical significance between the indicated groups was determined using unpaired, two-tailed Student's *t*-test.

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